

Appl. No. 09/730,329
Amdt. dated September 27, 2005
Reply to Office Action of June 27, 2005

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Amendments to the Specification:

Please replace paragraph [0060] with the following amended paragraph:

[0060] cDNA libraries were also screened using a 3'-RACE (Rapid Amplification of cDNA Ends) protocol according to methods well known in the art (White, B.A., ed., PCR Cloning Protocols; Humana Press, Totowa, NJ, 1997; ~~FIG. 9~~). Here primers derived from the 5' portion of the full length β -secretase gene are added to partial cDNA substrate clone found by screening a fetal brain cDNA library described above. A representative 3' RACE reaction used in determining the longer sequence is detailed in Example 3 and is described in more detail in Part B, below.

Please replace paragraph [0107] with the following amended paragraph:

[0107] In studies carried out in support of the present invention, isolated, purified forms of β -secretase were tested for enzymatic activity using one or more native or synthetic substrates. For example, as discussed above, when β -secretase was prepared from human brain and purified to homogeneity using the methods described in Example 5A, a single band was observed by silver stain after electrophoresis of sample fractions from the anion exchange chromatography (last step) on an SDS-polyacrylamide gel under reducing (+ β -mercaptoethanol) conditions. As summarized in Table 1, above, this fraction yielded a specific activity of approximately 1.5×10^9 nM/h/mg protein, where activity was measured by hydrolysis of MBP-C125SW. ~~FIG. 11 shows substrate concentration dependence of hydrolysis~~ Hydrolysis by brain β -secretase with respect to the Swedish mutant P13-P5' peptide, Swedish mutant P13-P5' with P1' aspartic acid replaced with an alanine (D \rightarrow A), a valine (D \rightarrow V), a phenylalanine (D \rightarrow F), a leucine (D \rightarrow L), and wild type APP shows substrate concentration dependence ~~(plotted at 10X amounts for visibility)~~. The valine and leucine substituted Swedish peptides show saturation at a lower concentration than the parent Swedish peptide. This likely indicates a higher binding affinity to the enzyme.

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Please replace paragraph [0153] with the following amended paragraph:

[0153] It was found that the P1 site is quite sensitive to substitution for binding with only Leu (the Swedish mutation substitution) and Phe showing appreciable cleavage (30%). Next P1 was allowed to remain as Leu and P1' was varied by substitution with different amino acids.

Interestingly, when P1' was varied there was quite a range of enzyme activity but when P1' was valine, the reaction kinetics showed that the enzyme became fully saturated. Thus this valine variant had good binding affinity for the protein as well as slowing the rate of cleavage of the bound complex.. An IC50 value of the 18 residue sequence in an MBP M125 assay was measured as 3 μ M, ~~3 μ M~~, which indicated an enzyme inhibition of over 100 times that of the Swedish sequence. This result demonstrated a clear effect on enzyme turnover. As a result, valine was selected as the amino acid substitution at P1' with further variation of P1 and more remote binding sides to be studied. When statine a non-standard amino acid known to inhibit renin, another aspartyl protease, was substituted at P1 with P1' being valine, a potent inhibitor of β -secretase was created. The P10-P4'sta D \rightarrow V inhibitor discussed above also incorporates this P1-Pi' sequence and is about 100 times more potent an inhibitor than the valine substitution alone.

Please replace paragraph [0187] with the following amended paragraph:

[0187] Poly A+ RNA from IMR human neuroblastoma cells was reverse transcribed using the Perkin-Elmer kit. Eight degenerate primer pools, each 8 fold degenerate, encoding the N and C terminal portions of the amino acid sequence obtained from the purified protein were designed (shown in Table 6; oligos 3407 through 3422). PCR reactions were composed of cDNA from 10 ng of RNA, 1.5 mM MgCl₂, 0.125 μ l AmpliTaq® Gold, 160 μ M each dNTP (plus 20 μ M additional from the reverse transcriptase reaction), Perkin-Elmer TAQ buffer (from AmpliTaq® Gold kit, Perkin-Elmer, Foster City, CA), in a 25 μ l reaction volume. Each of oligonucleotide primers 3407 through 3414 was used in combination with each of oligos 3415 through 3422 for a total for 64 reactions. Reactions were run on the Perkin-Elmer 7700 Sequence Detection machine under the following conditions: 10 min at 95°C, 4 cycles of, 45° C annealing for 15

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second, 72° C extension for 45 second and 95°C denaturation for 15 seconds followed by 35 cycles under the same conditions with the exception that the annealing temperature was raised to 55° C . (The foregoing conditions are referred to herein as "Reaction 1 conditions.") PCR products were visualized on 4% agarose gel (Northern blots) and a prominent band of the expected size (68 bp) was seen in reactions, particularly with the primers 3515-3518 in many of the lanes ~~(two gels were prepared, each of FIGS 3A-3C shows two gels, an upper and a lower gel, and the reaction combinations were run sequentially in the gels as illustrated, such that primer 3515 was reacted with each of 3507-3514, followed by reaction of primer 3516 with each of primers 3507-3514, and so forth).~~ The 68 kb band was sequenced and the internal region coded for the expected amino acid sequence. This gave the exact DNA sequence for 22 bp of the internal region of this ~~fragment.~~ fragment: C.GGC.CGG.AGG.GGC.AGC.TTT.GTG (SEQ ID NO:8).

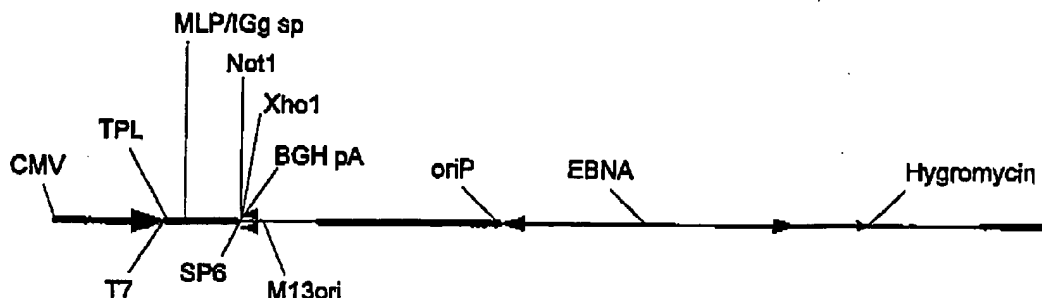
Please replace paragraph [0191] with the following amended paragraph:

[0191] A human primary neuronal cell library in the mammalian expression vector pCEK2 vector was generated using size selected cDNA, and pools of clones generated from different sized inserts. The cDNA library for β -secretase screening was made with poly(A)⁺ RNA isolated from primary human neuronal cells. The cloning vector was pCEK2 (map shown below).



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pCEK2

Please replace paragraph [0195] with the following amended paragraph:

[0195] PCR product was used as a substrate for random priming to generate a radiolabeled probe. 180,000 clones from the 1.5 kb pool (70,000 original clones in this pool), were screened by hybridization with the PCR probe and 9 positive clones identified. Four of these clones were isolated and by restriction mapping these appear to encode two independent clones of 4 to 5 kb (clone 27) and 6 to 7 kb (clone 53) length. Sequencing of clone 27 verified that it contains a coding region of 1.5 kb. See FIG. 13 for sequence of pCEK-clone27 (clone 27).

Table 6

| SEQ ID NO. | Pool No. | Nucleotide Sequence (Degenerate substitutions are shown in parentheses) |
|------------|----------|--|
| 13 | 3407 | G.AGA.GAC.GA(GA).GA(GA).CC(AT).GAG.GAG.CC |
| 14 | 3408 | G.AGA.GAC.GA(GA).GA(GA).CC(AT).GAA.GAG.CC |
| 15 | 3409 | G.AGA.GAC.GA(GA).GA(GA).CC(AT).GAA.GAA.CC |
| 16 | 3410 | G.AGA.GAC.GA(GA).GA(GA).CC(AT).GAG.GAA.CC |
| 17 | 3411 | AGA.GAC.GA(GA).GA(GA).CC(CG).GAG.GAG.CC |
| 18 | 3412 | AGA.GAC.GA(GA).GA(GA).CC(CG).GAA.GAG.CC |

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| | | |
|----|------|---|
| 19 | 3413 | AGA.GAC.GA(GA).GA(GA).CC(CG).GAA.GAA.CC |
| 20 | 3414 | AGA.GAC.GA(GA).GA(GA).CC(CG).GAG.GAA.CC |
| 21 | 3415 | CG.TCA.CAG.(GA)TT.(GA)TC.AAC.CAT.CTC |
| 22 | 3416 | CG.TCA.CAG.(GA)TT.(GA)TC.TAC.CAT.CTC |
| 23 | 3417 | CG.TCA.CAG.(GA)TT.(GA)TC.CAC.CAT.CTC |
| 24 | 3418 | CG.TCA.CAG.(GA)TT.(GA)TC.GAC.CAT.CTC |
| 25 | 3419 | CG.TCA.CAG.(GA)TT.(GA)TC.AAC.CAT.TTC |
| 26 | 3420 | CG.TCA.CAG.(GA)TT.(GA)TC.TAC.CAT.TTC |
| 27 | 3421 | CG.TCA.CAG.(GA)TT.(GA)TC.CAC.CAT.TTC |
| 28 | 3422 | CG.TCA.CAG.(GA)TT.(GA)TC.GAC.CAT.TTC |

Table 7

| SEQ ID NO. | Pool No. | Nucleotide Sequence |
|------------|----------|------------------------------------|
| 29 | 3458 | GAG GGG CAG CTT TGT GGA GA |
| 30 | 3468 | CAG.CAT.AGG.CCA.GCC.CCA.GGA.TGC.CT |
| 31 | 3469 | GTG.ATG.GCA.GCA.ATG.TTG.GCA.CGC |

Please replace paragraph [0198] with the following amended paragraph:

[0198] ~~FIG. 4 shows a schematic diagram of the specific~~ A 3'RACE strategy was used in experiments carried out in support of the present invention and effective to elucidate the polynucleotide encoding human β -secretase. Methods and conditions appropriate for replicating the experiments described herein and/or determining polynucleotide sequences encoding additional members of the novel family of aspartyl proteases described herein may be found, for example, in White, B.A., ed., PCR Cloning Protocols; Humana Press, Totowa, NJ, 1997, or Ausubel, *supra*, both of which are incorporated herein by reference.

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Please replace paragraph [0200] with the following amended paragraph:

[0200] The degeneracy of primers #3428 + 3433 was further broken down, resulting in primer set 2, comprising DNAs #3448-3455 (Table 3). Matrix RT-PCR was repeated using primer set 2, and cDNA reverse transcribed from poly-A+ RNA from IMR-32 human neuroblastoma cells (American Type Culture Collection, Manassas, VA), as well as primary human neuronal cultures, as template for amplification. Primers #3450 and 3454 from set 2 most efficiently amplified a cDNA fragment of the predicted size (72 bp), although primers 3450+3453, and 3450+3455 also amplified the same product, albeit at lower efficiency. The DNA sequence of the 72 bp PCR product obtained by amplification of cDNA from IMR-32 cells, and primary human neuronal cultures, with primers 3450 and 3454 was determined, ~~is shown in the lower portion of FIG. 4.~~

Please replace paragraph [0201] with the following amended paragraph:

[0201] Internal primers matching the upper (coding) strand for 3' Rapid Amplification of 5' Ends (RACE) PCR, and lower (non-coding) strand for 5' RACE PCR were designed and made according to methods known in the art (e.g., Frohman, M. A., M. K. Dush and G. R. Martin (1988). "Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene specific oligo-nucleotide primer." Proc. Natl. Acad. Sci. U.S.A. 85(23): 8998-9002.) The DNA primers used for this experiment were #3459 & #3460, ~~(#3459 & #3460)~~ are illustrated schematically in FIG. 4, and the exact sequences ~~sequence~~ of these primers are ~~is~~ presented in Table 3. These primers can be utilized in standard RACE-PCR methodology employing commercially available templates (e.g. Marathon Ready cDNA®, Clontech Labs), or custom tailored cDNA templates prepared from RNAs of interest as described by Frohman et al. (ibid.).

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Please replace paragraph [0238] with the following amended paragraph:

[0238] All manipulations were carried out at room temperature. 12.5 ml of 80% slurry of NHS-Sepharose (i.e. 10 ml packed volume; Pharmacia, Piscataway, NJ) was poured into a Bio-Rad EconoColumn (BioRad, Richmond, CA) and washed with 165 ml of ice-cold 1.0 mM HCl. When the bed was fully drained, the bottom of the column was closed off, and 5.0 ml of 7.0 mg/ml P10-P4'sta(D->V) peptide (dissolved in 0.1 M HEPES, pH 8.0) was added. The column was capped and incubated with rotation for 24 hours. After incubation, the column was allowed to drain, then washed with 8 ml of 1.0 M ethanolamine, pH 8.2. An additional 10 ml of the ethanolamine solution was added, and the column was again capped and incubated overnight with rotation. The column bed was washed with 20 ml of 1.5 M sodium chloride, 0.5 M Tris, pH 7.5, followed by a series of buffers containing 0.1 mM EDTA, 0.2% Triton X-100, and the following components; 20 mM sodium acetate, pH 4.5 (100 ml); 20 mM sodium acetate, pH 4.5, 1.0 M sodium chloride (100 ml); 20 mM sodium borate, pH 9.5, 1.0 M sodium chloride (200 ml); 20 , mM sodium borate, pH 9.5 (100 ml). Finally, the column bed was washed with 15 ml of 2 mM Tris, 0.01 % sodium azide (no Triton or EDTA), and stored in that buffer, at 4°C.

C. Sequences and inhibition data for heptamer peptides (Table 9)

with reference to the 10 peptide sequence around the β -secretase cleavage site of the Swedish mutation of APP (P5 to P5').

Table 9

| Example (Swedish) | P5 | | P3 | | P1 | | P1 | | P4 | | P5 | IC ₅₀ Group * |
|----------------------|----|---|------|-------|-------|-------|----|---|----|---|----|-----------------------------|
| | S | E | V | N | L | D | A | E | F | R | | |
| 7B | | | AcV- | -M- | -Sta- | -V- | - | - | - | - | | I |
| 7C | | | - | - | - | -Abu- | - | - | - | - | | II |
| 7D | | | - | - | - | -Phg- | - | - | - | - | | II |
| 7E | | | - | - | - | -A- | - | - | - | - | | II |
| 7F | | | - | - | - | t-Leu | - | - | - | - | | IV |
| 7G | | | - | -Phg- | - | -V- | - | - | - | - | | I |
| 7H | | | - | n-Leu | - | - | - | - | - | - | | I |
| 7I | | | - | -N- | - | - | - | - | - | - | | II |
| 7J | | | - | -F- | - | - | - | - | - | - | | II |
| 7K | | | - | -E- | - | - | - | - | - | - | | II |

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| | | | | | | | | | | |
|----|--|--|--------------------|-------|---------|-------|-------|-------|-------|----|
| 7L | | | ---- | -V-- | ----- | ----- | ----- | ----- | ----- | IV |
| 7M | | | ---- | -G-- | ----- | ----- | ----- | ----- | ----- | II |
| 7N | | | ---- | --M-- | ----- | xxxx† | ----- | ----- | ----- | II |
| 7O | | | ---- | ----- | phe-Sta | -V-- | ----- | ----- | ----- | I |
| 7P | | | ---- | ----- | nor-Sta | ----- | ----- | ----- | ----- | II |
| 7Q | | | (NH ₂) | ----- | acha | ----- | ----- | ----- | ----- | II |
| 7R | | | ---- | ----- | -Sta-- | ----- | xxxx | ----- | ----- | II |

Heptamers are mapped as P3 to P4'. A dashed line indicates the amino acid at that position is the same as the one immediately above it. A vertical line in a box indicates that that amino acid is the N- or C-terminus.

* Group I has IC₅₀ of <1 μ M; Group II of =1 to <10 μ M; Group III =10 to <20 μ M; Group IV =20 to <50 μ M.

† indicates that the amino acid was deleted from the sequence making it a hexameric peptide
 Abu = α -aminobutyric acid; Phg = phenylglycine; t-Leu = tertiary leucine; n-Leu is normal leucine; Statine is a known γ -amino acid Merck Index 11th Ed, # 8759 having the formula (CH₃)₂CHCH(NH₂)CH(OH)CH₂CO₂H, phe-Sta = phenylstatine (i.e. Sta having a benzyl side chain); acha is cyclohexylstatine (i.e. Sta having a cyclohexylmethyl side chain); nor-Sta is the statine residue without the methylene group intervening between the carbonyl and hydroxymethylene groups, effectively it is Sta with the "backbone" shortened by one methylene group.

Please replace paragraph [0241] with the following amended paragraph:

[0241] 293T cells were co-transfected with equivalent amounts plasmids encoding APP^{sw} or wt and β -secretase or control β -galactoside (β -gal) cDNA using FuGene 6 Reagent, as described in Example 4, above. Either pCEKclone27 or pohCJ containing full length β -secretase were used for expression of β -secretase. The plasmid construct pohCK751 used for the expression of APP in these transfections was derived as described in Dugan et al., JBC, 270(18) 10982-10989(1995) and shown in FIG. 21. A β -gal control plasmid was added so that the total amount of plasmid transfected was the same for each condition. β -gal expressing pCEK and

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pohCK vectors do not replicate in 293T or COS cells. Triplicate wells of cells were transfected with the plasmid, according to standard methods described above, then incubated for 48 hours, before collection of conditioned media and cells. Whole cell lysates were prepared and tested for the β -secretase enzymatic activity. The amount of β -secretase activity expressed by transfected 293T cells was comparable to or higher than that expressed by CosA2 cells used in the single transfection studies. Western blot assays were done on conditioned media and cell lysates, using the antibody 13G8, as described below, and A β ELISAs carried out on the conditioned media to analyze the various APP cleavage products.